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Methods of sample preparation of radula epithelial tissue in chitons (Mollusca: Polyplacophora)*

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Abstract: A glutaraldehyde fixative developed for preserving the radula superior epithelium of the adult chiton *Acanthopleura hirtosa* (Blainville, 1825), was used in conjunction with conventional and microwave-assisted sample processing to produce high quality tissue preservation for light and electron microscopy. In addition, high-pressure freezing (HPF) and cryo-substitution were used to fix the radula tissue of juvenile specimens. Microwave-assisted fixation was preferred to conventional bench-top techniques due to the superior preservation of fine cell structure together with reduced processing times and chemical exposure. Although restricted to very small (<200 μm) samples, the quality of juvenile radulae processed by HPF was excellent. The improvements in tissue preservation using microwave and cryo-preservation techniques are therefore critical for obtaining accurate ultrastructural information on the radula in marine molluscs. In particular, these findings highlight additional processing options available for the study of cellular structures in biomineralizing tissues.

Key words: microwave, high-pressure freezing, chemical fixation, cryo-fixation, biomineralization

The radula has been the focus of numerous studies over many decades, with its intricate and varied design used to elucidate aspects of molluscan ecology, biology, and taxonomy (Fretter and Graham 1962, Runham 1963, Steneck and Watling 1982, Padilla 1985, Scheltema 1988, Salvini-Plawen 1990). In addition, the radulae of polyplacophoran and patellid gastropods have received particular attention as a result of their unique ability to harden their teeth with iron and other biominerals (Mann *et al.* 1986, Lowenstam and Weiner 1989, Webb *et al.* 1989).

The chiton radula represents an excellent example of matrix-mediated biomineralization, where minerals are formed in a highly organized manner within the framework of an organic matrix (Simkiss and Wilbur 1989, Watabe 1990, Mann 2001, Weiner and Addadi 2002). While considerable progress has been made in elucidating the general structural organization of minerals deposited within the tooth matrix and the sequence in which they are deposited (Lowenstam 1962, Kim *et al.* 1989, Macey *et al.* 1994, Brooker *et al.* 2006), the mechanisms involved in cellular transport of ions to the tooth cusps are poorly understood. The elemental precursors for biomineralization are thought to be delivered to the teeth by the overlying, superior epithelial tissue, which surrounds the cusps during all stages of development (Nesson and Lowenstam 1985) (Fig. 1). The superior epithelium and teeth are encapsulated within the radular sheath, the inferior epithelium, and the radula membrane with the whole structure resembling a tube open only along the dorsal surface (Fig. 1). Histological investigation of epithelial tissue is difficult due to the complex composition and structure of the radula, which contains both hard mineralized structures and cartilaginous membranes in close proximity to cellular material.

Chemical fixatives such as glutaraldehyde buffered in filtered seawater are commonly used for preserving marine organisms, where the osmotic pressure of the solution acts to mimic that of the animal, thereby reducing swelling or shrinkage of the tissues (Dykstra and Reuss 2003). However, fixation often gives rise to variations in the ultrastructural morphology of organelles (Hayat 2000), and it is therefore

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preferable to utilize several techniques to acquire comparative information. Microwave-assisted fixation and cryo-preservation techniques (McDonald *et al.* 2007, Webster 2007), now common in laboratories, provide two additional means of obtaining such comparative information. The main advantage of microwave protocols is a dramatic reduction in sample processing times (<4 hours), while at least maintaining, if not improving, fixation quality over conventional methods (Giberson and Demaree 1999, Laboux *et al.* 2004). Excellent tissue preservation can also be attained using cryo-preservation techniques, which achieve vitreous ice formation in samples and thereby prevent ice crystal damage. However, due to difficulties associated with heat dissipation, only very small samples (<200 μm) can be frozen successfully (Wilson *et al.* 1998, Sawaguchi *et al.* 2005).

To improve our current techniques and better understand the detailed cellular structure of the superior epithelium in chiton and limpet teeth, we investigated alternative fixation methods. Determining the precise structure of these cells will assist in elucidating their function and the mechanisms involved in the transport of ions into the teeth, a fundamental obstacle to our wider understanding of the initial phase of biomineralization. The aim of this study is to compare three fixation methodologies (conventional chemical, microwave-assisted, and low temperature), regarding preservation of the superior epithelial tissues of the chiton *Acanthopleura hirtosa* (Blainville, 1825) for both light and electron microscopy.

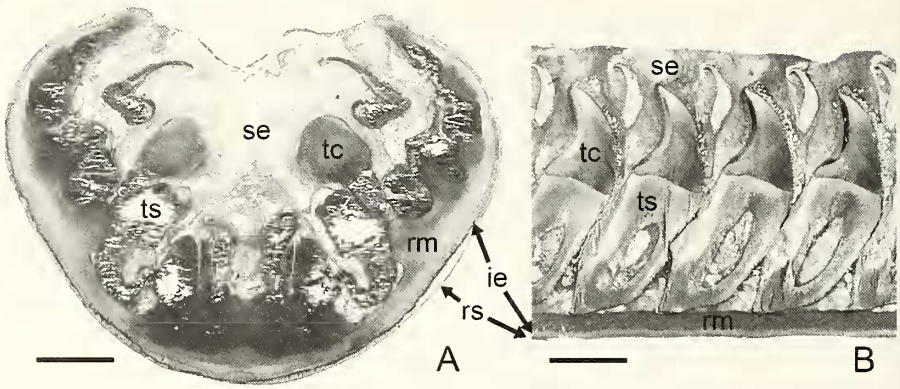

Figure 1. Radula apparatus of the chiton *Acanthopleura hirtosa* (adult) in (A) transverse section and (B) longitudinal section exhibiting various hard and soft tissue components. Abbreviations: se, superior epithelium; ie, inferior epithelium; tc, tooth cusp; ts, tooth stylus; rm, radula membrane; rs, radula sheath. Scale bars = 200 μm.

MATERIALS AND METHODS

Conventional and microwave-assisted chemical fixation and cryo-fixation techniques were utilized for the preparation of epithelial tissues for light microscopy (LM) or transmission electron microscopy (TEM). Adult and juvenile specimens of the chiton *Acanthopleura hirtosa* (mean animal length ~4 cm and ~0.8 cm, respectively) were collected from intertidal limestone at Woodman Point within the Perth metropolitan area of Western Australia (32°08′S, 115°44′E). Incisions were made along both pallial grooves from the anus towards the head, thereby freeing the foot, visceral mass, and buccal mass as a single entity from the shell plates and girdle. The visceral mass was then carefully removed to expose the radula sac. Dissections were performed as quickly as possible to reduce the deterioration of radula epithelial tissue.

Table 1. Conventional and microwave-assisted processing schedules for the radula epithelial tissues of adult *Acanthopleura hirtosa.*

Step	Medium	Concentration (%)	Conventional times	Microwave times	Microwave wattage (W)
Fixation	glutaraldehyde (buffer A)	2.5*	24	$2\times(2^{on}/2^{off}/2^{on})$(min)(v)	80
Rinse	buffer A		15 min ×4	40 sec (v)	250
Post-fixation	OsO_4 (buffer B)	1*	2 h	$2\times(2^{on}/2^{off}/2^{on})$(min)(v)	80
Rinse	buffer B		15 min ×4	40 sec (v)	250
Dehydration	acetone	10, 30, **50***, **75***, **90***, **100***	15 min ×2 each	40 sec each (100 ×2)	250
Infiltration	Spurr's resin	5, 10, 20, 40, **50**, 60, **75**, 80, **90, 100***	8-12 h each	3 min each (100 ×2) (v)	250
Polymerization	Spurr's resin	**100***	conventional oven 60 °C overnight		

Note: Concentrations in bold represent the microwave schedule, concentrations not in bold represent the conventional schedule, and * represents concentrations used in both schedules.

(v) = steps undertaken in vacuum

$^{on/off}$ denotes magnetron (irradiation) cycle

Table 2. Conventional and microwave-assisted processing schedules for the radula epithelial tissues of juvenile *Acanthopleura hirtosa*.

Step	Medium	Concentration (%)	Conventional times	Microwave times	Microwave wattage (W)
Fixation	glutaraldehyde (buffer A)	2.5*	24 h	$2\times(2^{on}/2^{off}/2^{on})$ (min) (v)	80
Rinse	buffer A		10 min ×4	40 sec (v)	250
Post-fixation	OsO_4 (buffer B)	1*	1 h	$2\times(2^{on}/2^{off}/2^{on})$ (min) (v)	80
Rinse	buffer B		10 min ×4	40 sec (v)	250
Dehydration	ethanol/acetone[(a)]	**50*, 75*, 90*, 100*, 100**[(a)], 100[(a)]	10 min each (100 ×2)	40 sec each	250
Infiltration	Procure Araldite	5, 20, **50**, 60, **75**, 80, **90, 100***	4-8 h each	3 min each (100 ×2) (v)	250
Polymerization	Procure Araldite	**100***	conventional oven 60 °C overnight		

Note: Concentrations in bold represent the microwave shecule, concentrations not in bold represent the conventional schedule, and * represents concentrations used in both schedules.

(v) = steps undertaken in vacuum

$^{on/off}$ denotes magnetron (irradiation) cycle

$^{(a)}$ = acetone

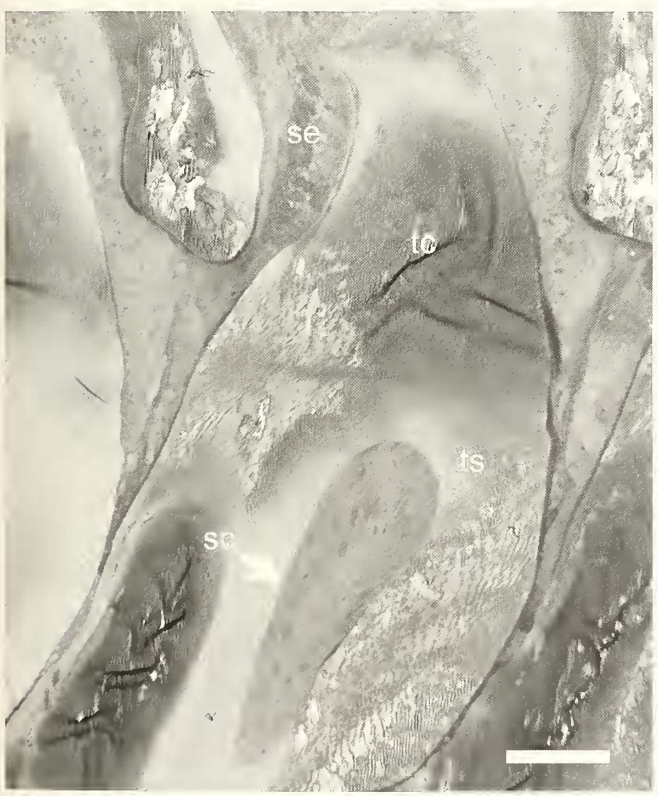

Figure 2. Light micrograph of a longitudinal section through a major lateral tooth from adult *Acanthopleura hirtosa* at row 6 prepared using microwave-assisted protocols. Despite being situated deep within the tooth stylus (ts), the tissues of the stylus canal (sc) are well preserved. se, superior epithelium; tc, tooth cusp. Scale bar = 50 μm.

Preparation of radulae from adult animals

For samples processed using either conventional or microwave-assisted methods, the dissected tissue mass was immediately immersed in a fixative comprised of 2.5% glutaraldehyde buffered in 0.1 M phosphate, with a pH of 7.2 and an osmolarity of 900 mmol kg^{-1} adjusted using sucrose (buffer A). The buccal mass and radula were separated from the remainder of the animal, and the radula was either left whole or cut transversely into three or four segments (the buccal mass was discarded). The tissues were then processed by either conventional bench-top methods or accelerated microwave-assisted protocols using a Pelco Biowave® fitted with a cold spot and vacuum chamber, according to the specific schedules detailed in Table 1. This included fixation with glutaraldehyde in buffer A, rinsing in buffer A, post fixation in 1% osmium tetroxide (OsO_4) in 0.05 M phosphate buffered saline (buffer B) at 4 °C, and a final rinse in buffer B, prior to dehydration through a graded series of acetones, then infiltration and embedding in Spurr's resin. Preparation of radula tissue by high-pressure freezing was not possible for adults due to limitations in sample size, which was again restricted to ~200 μm.

Following polymerization, resin blocks were sectioned for observation at both the LM and TEM level. For LM, 1 μm-thick sections were mounted on glass slides and stained with aqueous 1% Methylene Blue and 1% Azur II (20 sec) prior to imaging on an Olympus BX51 optical microscope fitted with an Olympus DP70 digital camera. For TEM, ~60 nm-thick sections were mounted on copper grids and double stained with uranyl nitrate (single crystal in one drop of 50% methanol) (10 min) followed by Sato's modified lead citrate (10 min) (Hanaichi *et al.* 1986) prior to imaging on a JEOL 2000 TEM at 80 kV.

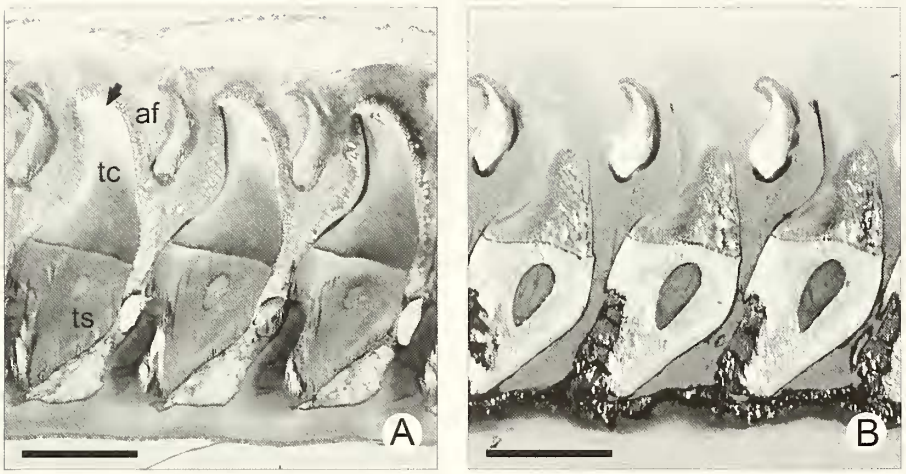

Figure 3. Major lateral tooth rows 12-14 from radulae of adult *Acanthopleura hirtosa* prepared using (A) conventional and (B) microwave techniques. Note the separation (arrow) of the superior epithelium from the anterior face (af) of the tooth cusps (tc) in conventionally fixed material and the poor infiltration of resin into the tooth stylus (ts) and cusp in microwave prepared material. Scale bars = 200 μm.

Preparation of radulae from juvenile animals

Juvenile specimens of *Acanthopleura hirtosa* were processed similarly using conventional and microwave-assisted methods; however, schedules were adjusted in order to account for the reduction in tissue size, and samples were embedded in Procure Araldite (formerly Epon Araldite) (Table 2). For cryo-fixation of juvenile radulae, the immature portion of each radula was removed by cutting transversely approx. 200 μm from the posterior end. Each immature portion was then placed into a 200 μm membrane filled with 20% bovine serum albumin in artificial seawater. These membrane-mounted samples were rapidly frozen in a high-pressure freezer (Leica EMPACT 2) prior to cryo-substitution in acetone containing 2% OsO_4 at −85 °C for 52 h. Samples were then progressively warmed from −85 °C to 20 °C over 13 h in a Leica automatic freeze substitution unit prior to being washed in acetone and infiltrated and embedded in Araldite resin. Cryo-prepared sample blocks were polymerized underwater using a Pelco Biowave® microwave at 650 W for 90 min. Conventional, microwave-assisted and cryo-prepared sections were imaged unstained on a JEOL 2100 TEM at 120 kV, using a Gatan Orius SC1000 digital camera.

RESULTS AND DISCUSSION

Preservation of adult radulae

While glutaraldehyde fixation of radula epithelium was satisfactory at the LM level when conventional bench-top methods were used, microwave-assisted protocols dramatically reduced sample processing times from six days to one hour (Table 1) and resulted in superior ultrastructural preservation at the TEM level. In addition, microwave-assisted protocols increased fixative penetration into the tooth, improving, for example, tissue preservation within the stylus canal (Fig. 2), which fixed poorly by conventional methods.

The endothelial radula sheath layer, recognizable by the presence of ciliated endothelial cells distributed over the entire sheath's surface, remained intact when processed by microwave methods (Fig. 1), in contrast to conventional methods where it was often disrupted. It is likely that the shorter sample processing and handling times with microwave fixation reduce the likelihood of damage to this delicate membrane. Both techniques preserved prominent vesicles that line the anterior and posterior surfaces of the tooth cusps (Fig. 3). We have found that these vesicles can be either abundant or virtually absent at the same stage of tooth development in different animals. It is currently not known whether these vesicles are natural features of the adult epithelium or artifacts resulting from the fixation process.

Conventional fixation often resulted in the separation of the superior epithelium from the hard tooth cusps, while in microwave-fixed material this artifact was rarely observed (Fig. 3). Despite a number of attempts to improve resin penetration into the base material using both conventional and microwave-assisted methods, including lower resin concentrations and increased infiltration times, the fibrous appearance of the major lateral tooth cusps and stylus persisted and was indeed far more noticeable in microwave-prepared specimens (Fig. 3). This is indicative of poor infiltration by the epoxy resin and is a common problem encountered in mollusc species by many researchers (Nesson and Lowenstam 1985, Mackenstedt and Markel 1987).

Microwave-assisted sample processing resulted in far better preservation of tissue ultrastructure compared to conventional methods. TEM of both conventional and microwave-prepared samples revealed the typical arrangement of organelles within the superior epithelium near the cusp surface that are common to chitons, including microvilli, mitochondria, rough and smooth endoplasmic reticulum, and abundant, electron-dense ferritin siderosomes (Fig. 4). However, at higher magnifications it could be clearly seen

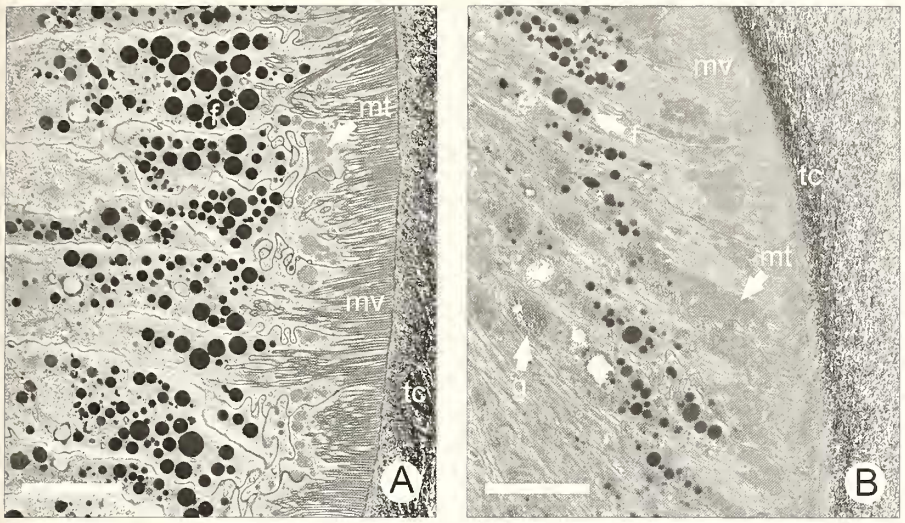

Figure 4. Transmission electron micrographs of the radula superior epithelium abutting the tooth cusp (tc) of (A) conventionally and (B) microwave-processed radulae from adult *Acanthopleura hirtosa* at tooth rows 14 and 13, respectively. f, ferritin siderosome; g, granules; mt, mitochondria; mv, microvilli. Scale bars = 5 μm.

that samples fixed using the microwave contained numerous granules approx. 60 nm in diameter. These granules, which are likely to be either aggregations of ribosomal material or glycogen, appeared throughout the cytoplasm, particularly near the apical poles of the superior epithelium (Fig. 5). These fine structures are either absent or not well preserved in conventional preparations and have not been reported by previous authors (Nesson and Lowenstam 1985, Kim *et al.* 1989). It is likely that the retention of these structures in microwave-prepared samples is a result of the dramatic reduction in processing time of samples, thereby reducing chemical exposure and the chance of extracting soluble components of the tissue.

Preservation of juvenile radulae

The type and arrangement of organelles within the apical epithelium of juvenile *Acanthopleura hirtosa* radulae follows the same characteristic configuration as that described for adult tissue. The preservation quality of juvenile radula epithelium, when using conventional and microwave-assisted methods, was also very similar to that observed in adults (Figs. 6A-B). While the reduction in sample size may improve fixative penetration in juvenile radulae, the comparable fixation quality between adult and juvenile tissue indicates that size is not a limiting factor. In support of this, the ~60 nm granules were absent in conventionally fixed juvenile tissue but were retained in adult and juvenile tissue when processed by the microwave protocol (Figs. 5B, 6B).

With the exception of slight separation of epithelial tissue from the tooth cusps and bases, high-pressure freezing (HPF) resulted in unsurpassed preservation of juvenile *Acanthopleura hirtosa* radula tissue (Fig. 6C). Nuclei (with well defined chromatin adjacent to the nuclear membrane), mitochondria (with well preserved cristae), rough endoplasmic reticulum, and Golgi apparatus were clearly represented in the cytoplasm, together with microvilli and ferritin siderosomes (Fig. 6C). The ~60 nm granules observed in microwave-assisted preparations were also present in HPF samples (data not shown). The large vesicles surrounding the tooth cusps in adult epithelium were also observed in juvenile tissue prepared using conventional and microwave protocols but were absent in HPF material (data not shown). While variations in

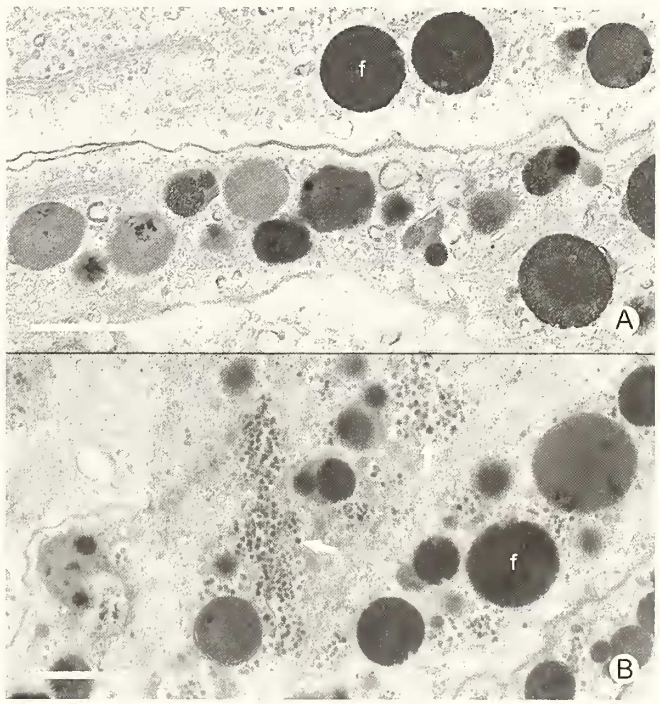

Figure 5. Transmission electron micrograph of (A) conventional and (B) microwave-prepared superior epithelium from adult *Acanthopleura hirtosa* showing differences in the preservation of fine structure. Arrows denote aggregations of ribosomal- or glycogen-like structures within the cytoplasm. f, ferritin siderosome. Scale bars = 1 μm.

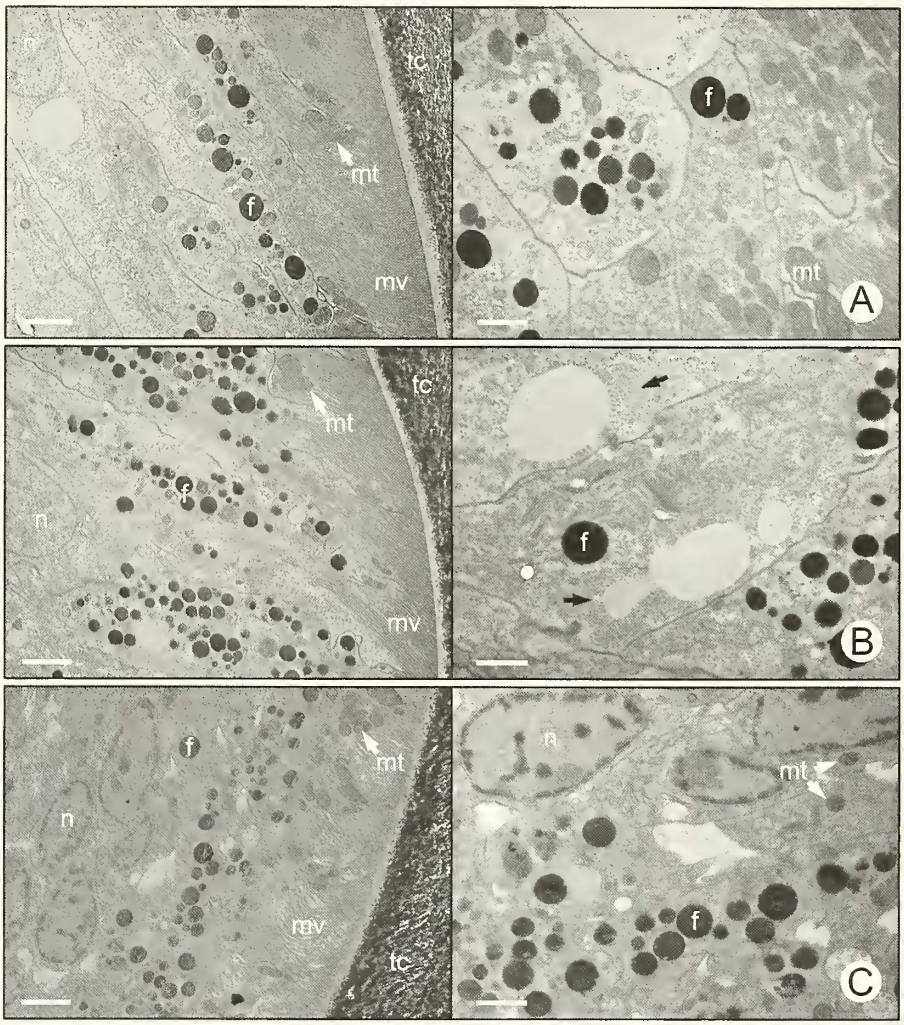

Figure 6. Transmission electron micrographs comparing the preservation of cell ultrastructure in radula epithelium from juvenile specimens of the chiton *Acanthopleura hirtosa* processed using (A) conventional, (B) microwave-assisted, and (C) high-pressure freezing protocols. Black arrows denote aggregations of ribosomes/glycogen. f, ferritin siderosome; mv, microvilli; mt, mitochondria; n, nucleus; tc, tooth cusp. Note: all images were taken from the first tooth row after the onset of mineralization in the cusps. Scale bars = 2 μm (images on left) and 1 μm (images on right).

ultrastructure may arise from differences in the functional state of the cells at the time of fixation (Hayat 2000), it is more likely that these vesicles are artifacts resulting from glutaraldehyde fixation (Bowers and Maser 1988).

Despite a slight improvement in resin infiltration using the conventional method, microwave-assisted processing of radula superior epithelium is preferred due to the improved quality of tissue preservation and the vastly reduced time for sample processing. While cryo-preservation using HPF results in excellent ultrastructural preservation, it is limited with respect to sample size. As such, only the radulae of juveniles or very small mollusc species can be prepared using this method. In addition, the relative portability and affordability of microwave technology compared to HPF makes it a more realistic option for many laboratories. While each of these new techniques has proven to be suitable for fixation of tissue at the LM level, the improved retention of ultrastructural information gained by using microwave and HPF methods highlights the need for a re-evaluation of fine cell structure in molluscan radulae.

While the ultrastructure of the radula epithelium of chitons and limpets has been well documented (Nesson and Lowenstam 1985, Mann *et al.* 1986, Kim *et al.* 1989, Rinkevich 1993), no studies have been conducted on the development of this tissue as teeth progress from an unmineralized to a mineralized state, information that is crucial for resolving the cellular basis of biomineralization. In addition, cryo-techniques have recently been used in conjunction with chemical fixation to characterize the organic matrix in limpets, by dramatically reducing artifacts resulting from staining, dehydration, and embedding (Sone *et al.* 2007). The high-pressure freezing method outlined in the current study therefore provides a valuable first step in preserving the organic matrix for subsequent cryo-sectioning in a frozen hydrated state. The methods of sample preparation presented here not only will benefit future investigations of the superior epithelium and organic matrix of chitons and limpets but also will be of use for taxonomic and morphological studies of molluscan radulae in general.

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